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TITLE: A Global Quantitative Analysis of Operon/Gene Transcription in *Bacillus anthracis* During Experimental Infection

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The objective is to perform the first comprehensive quantitative analysis of *Bacillus anthracis* operon/gene transcription during *in vivo* experimental infection to enable the identification of additional virulence factors, potential vaccine candidates, and novel targets for therapeutic intervention. This study is planned to identify genes of interest by examining gene transcription in *B. anthracis* under several experimental conditions. This includes comparing in vitro vs. in vivo grown organisms to identify those genes expressed exclusively in the host. Secondly, to identify those genes specifically expressed early after germination compared to those expressed during the terminal stages of infection. This will hopefully identify genes and targets that are specific to the earliest stage of the infectious process. The genes will be identified using quantitative RT-PCR by the Taqman assay.

1. Bioinformatic analysis of the *B. anthracis* genome has been completed and approximately 1230 ORFs have been selected for study and primer/probe sets have been obtained.
2. Analysis of gene expression during early stage *B. anthracis* infection requires delivery of $10^8$ spores intradermally followed by a 2 hour incubation during which significant spores germinate but outgrowth of the bacilli is minimized.
3. Empirical optimization of tissue handling, RNA extraction, cDNA synthesis, and Taqman assay conditions has significantly enhanced our ability to measure *B. anthracis* gene expression within host tissues.
4. Pre-amplification of cDNA derived from early-stage infected tissues will permit analysis of *B. anthracis* gene expression at this critical phase of infection.
5. cDNA from late stage infection in the guinea pig has been prepared.
6. cDNA from early stage infection is being isolated.
7. Upon receipt of nested primers, the comparison of gene expression during in vitro cultivation vs. early and late stage infection will begin.

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## INTRODUCTION:

This study is planned to identify genes of interest by examining gene transcription in *B. anthracis* under several experimental conditions. This includes comparing in vitro vs. in vivo grown organisms to identify those genes expressed exclusively in the host. Secondly, to identify those genes specifically expressed early after germination compared to those expressed during the terminal stages of infection. This will hopefully identify genes and targets that are specific to the earliest stage of the infectious process. The genes will be identified using quantitative RT-PCR by the Taqman assay. This will be the first comprehensive quantitative analysis of *Bacillus anthracis* operon/gene transcription during *in vivo* experimental infection to enable the identification of additional virulence factors, potential vaccine candidates, and novel targets for therapeutic intervention.

## BODY:

Bioinformatic Analysis:

The first stage of this process was to perform a bioinformatic analysis of the B. anthracis genome using the latest information obtained from the TIGR annotation. This was necessary because the funds provided only enabled analysis of a subset of the entire genome. The criteria for ORF selection included secreted and membrane associated genes, those unique to *B. anthracis* after comparison with other Bacillus genomes, genes identified to be missing from attenuated strains of *B. anthracis* and those with homology to selected known virulence factors. This resulted in a list of approximately 1230 ORFs. Primer/probe sets for all ORFs have now been obtained.

RNA Extraction Procedures and Experimental Infection

In vitro:

Procedures for extraction of mRNA and optimizing sensitivity of quantitative RT-PCR have been determined for the in vitro grown organisms.

In vivo:

Extensive experiments performed in the guinea pig model identified by histological analysis the earliest time when spores germinated but there was still limited bacterial proliferation. This was critical to establish because it will allow us to focus on the earliest outgrowth of spore to bacillus. Analysis of gene expression during early stage *B. anthracis* infection requires delivery of $10^8$ spores intradermally followed by a 2 hour incubation during which significant spores germinate but outgrowth of the bacilli is minimized. With this information, to date our efforts and progress have focused on optimization of several technical issues that directly affect our ability to measure *B. anthracis* gene expression in host tissues. While the initial extraction procedures were sufficiently sensitive for the amounts of bacteria present during the terminal stage of infection, they proved insufficiently sensitive to detect genes expressed at lower levels during the early stage of infection. Technical issues addressed include method and empirical optimization of tissue processing and handling, RNA extraction from the bacillus present in host tissue, generation of cDNA, as well as Taqman assay composition and conditions for our system. These significant advances, which are summarized below, are critical for the success of the study and will enable completion of our goal of identifying *B. anthracis* genes induced specifically within host tissues that may be essential for virulence of the pathogen.

Optimization of tissue processing and handling. The method of RNA extraction employed requires that *B. anthracis* infected tissues be processed by dissection into 100 mg samples for RNA extraction. Initially, processed tissues were flash frozen in a dry-ice methanol bath and stored frozen in a –70°C freezer. No signals were obtained in these samples when analyzed using a Taqman probe for a demonstrated *B. anthracis* virulence gene (*pagA,* which encodes protective antigen). This observation provided the impetus to optimize several aspects of sample preparation in order to generate signals in the Taqman assay and permit measurement of *B. anthracis* gene expression in infected tissues. Prolonged handling and thawing of frozen samples is known to permit degradation of messenger RNA and reduce signals; therefore, an agent that stabilizes RNA (RNA Later, Ambion) was used for tissue processing and storage. The utility of this reagent was demonstrated as *pagA* Taqman signals were significantly and reproducibly enhanced ($C_t$ of 37-41) from the treated samples while no or reduced signal was observed for untreated samples.

Optimization of RNA extraction. Extraction of RNA from *B. anthracis* grown in laboratory culture as well as infected tissues employs reagents and an instrument specifically designed to disrupt Gram-positive bacteria and yeast. Optimization of the extraction procedure was accomplished by varying the duration of mechanical bacterial disruption in the instrument and determining efficiency of RNA extraction by Taqman analysis. We found that extraction of RNA from infected tissues required significantly different conditions than bacteria grown in laboratory medium. Essentially equivalent Taqman signals for the 16S rRNA locus were obtained from infected tissue samples

incubated 15, 30, 60, and 120 seconds in the instrument. In contrast, Taqman signals for the same locus were maximal at 60 and 120 seconds indicating that bacteria cultured in vitro require longer incubation to thoroughly disrupt the cells. These findings define conditions used for RNA extraction and are integral to success of our approach as they maximize our ability to measure *B. anthracis* gene expression in infected tissues.

Optimization of cDNA synthesis. Generation of cDNA from total RNA isolated from infected tissues employs a DNase step (to eliminate contaminating host and bacterial genomic DNA) followed by a reverse transcriptase (RT) reaction in which cDNA is synthesized from the RNA. We have modified several aspects of the DNase and RT reaction including addition of ribonuclease inhibitors (a ubiquitous enzyme that rapidly degrades RNA). We have also modified the duration of the DNase reaction (from 1 hour to 20 minutes) in an effort to minimize conditions conducive to RNase activity. Each of these modifications has contributed to Taqman signal enhancement for housekeeping (*rpoB, era,* 16S rRNA) and virulence (*pagA*) loci.

Optimization of Taqman assay conditions. Initially, our Taqman assay employed a general enzyme master mix which included components designed to minimize reaction contamination (Supermix-UDG). Signals generated using this reagent were determined for a number of loci and, though the mix performed as expected, we believed that components that reduce risk of contamination (UTP and uracil deglycosylase) might significantly inhibit polymerase activity, particularly when the substrate is an A-T rich template such as cDNA generated from *B. anthracis* messenger RNA. Thus a custom master mix (no UTP or uracil deglycosylase) was obtained and a comparison was made with the standard mix in the Taqman assay using *B. anthracis* genomic DNA as template (the cDNA is A-T rich, like the genomic DNA from which it is derived). Taqman signals were dramatically enhanced for 16S rRNA suggesting that omission of UTP from the reaction significantly enhances polymerase activity. These modifications, in conjunction with changes to tissue handling, RNA extraction and cDNA synthesis methods have significantly enhanced detection of genes expressed within the host as determined by Taqman analysis. For example, Taqman signals generated from RNA extracted from early-stage infected tissues for 16s rRNA have been significantly enhanced (from $C_t$ 39 to $C_t$ 22) using these optimizations.

Analysis of medium to low expressed genes. Unfortunately, results showed that Taqman signals for medium and low-expressed genes have been extremely low or undetectable and not reproducible even after incorporation of the various optimization steps outlined above. To overcome this obstacle we evaluated the use of nested primers to enhance signals generated from these loci. Pre-amplification of cDNA prior to the Taqman assay significantly and reproducibly enhanced *rpoB* signals from our most challenging infection model, the early stage infection. As a result nested primers have been ordered for all loci targeted in this study and it is anticipated that this added step will permit analysis of *B. anthracis* gene expression during the critical early stages of infection.

## KEY RESEARCH ACCOMPLISHMENTS:

1. Bioinformatic analysis of the *B. anthracis* genome has been completed and approximately 1230 ORFs have been selected for study and primer/probe sets have been obtained.
2. Tissue handling, RNA extraction, cDNA synthesis, and Taqman assay conditions have been optimized to significantly enhance our ability to measure *B. anthracis* gene expression within host tissues.
3. cDNA from late stage infection in the guinea pig has been prepared.

## REPORTABLE OUTCOMES:

None at this point.

## CONCLUSIONS:

Technology transfer from GSK to USAMRIID has been completed with *B. anthracis* ORF selection, receipt of TaqMan probe/primer sets and reagents and optimization of animal models, RNA extraction and RT-PCR conditions.

The following experiments remain to complete this work:

1. Generation of cDNA from early stage infection (in progress).
2. Preamplification of cDNA derived from early-stage infected tissues to permit analysis of *B. anthracis* gene expression at this critical phase of infection.
3. Comparison of gene expression during *in vitro* cultivation vs. early and late stage infection.